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Effects of eating disorders on oral fungal diversity

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Background. The eating disorders anorexia and bulimia nervosa can cause several systemic and oral alterations related to poor nutrition and induced vomiting; however, the oral microflora of these patients is poorly studied.
Objective. The aim of this study was to evaluate fungal microflora in the oral cavity of these patients by culture-dependent and culture-independent methods.
Study Design. Oral rinse samples were cultured to assess the prevalence of *Candida* species, and the isolates were identified by API system. Microorganism counts were compared by the Mann-Whitney test (5%). Ribotyping, a type of molecular analysis, was performed by sequencing the D1/D2 regions of 28S rRNA.
Results. Our results demonstrated that the eating disorder group showed higher oral *Candida* spp. prevalence with culture-dependent methods and higher species diversity with culture-independent methods.
Conclusions. Eating disorders can lead to an increased oral *Candida* carriage. Culture-independent identification found greater fungal diversity than culture-dependent methods. (Oral Surg Oral Med Oral Pathol Oral Radiol 2012;113:512-517)

The prevalence of eating disorders (EDs) has increased significantly over the years, mainly stimulated by a modern society that upholds thinness as a symbol of success and beauty.[1] Anorexia nervosa (AN) is characterized by weight loss at the expense of an extremely restricted diet, an unbridled quest for thinness, a distorted body image, and menstrual cycle alterations. Bulimia nervosa (BN) is characterized by binge-eating (quickly eating large quantities of food with little or no pleasure) alternating with behaviors to prevent weight gain.[1,2] Although classified separately in the Diagnostic and Statistical Manual edition IV (DSM-IV),[3] these 2 disorders are closely related because they have a common psychopathology: excessive concern about weight and body shape (morbid fear of fatness).[4] EDs are accompanied by various clinical complications related to impaired nutritional status and unhealthy compensation practices for weight control, such as induced vomiting or the use of diuretic and laxative drugs.[4,5] Morbidity and mortality rates are notably high. AN has the highest mortality rate among all psychiatric disorders (~0.56% per year), which is about 12 times greater than the mortality rate of young women in the general population.[6]

Oral manifestations include a number of signs involving the oral mucosa (mucosal atrophy, erythematous lesions, and opportunistic infections), teeth (perimolysis and caries), periodontium (gingivitis and periodontitis), and salivary glands (sialadenosis and hyposalivation). EDs may also be associated with a number of oral symptoms, such as a burning sensation, dental sensitivity, dysgeusia, and xerostomia. These signs and symptoms are caused by a number of factors, including nutritional deficiencies with consequent metabolic impairment, poor personal hygiene, use of drugs, vomiting, modified nutritional habits and underlying psychologic disturbances.[2,7]

Oral microflora is relatively stable in healthy individuals, but systemic and/or local changes may disrupt the biologic balance between the microbiota and the host.[8] Oral candidiasis is an opportunistic infection and

Statement of Clinical Relevance

Although fungi are important components of oral microbiota, studies have focused on oral bacteria. We focused on oral fungi flora from eating disorders patients, owing to reports of lesions and systemic conditions in which fungi may be related.[11]

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has been frequently reported in eating disorders (ED) patients.[9,10] However, little is known about the oral microflora in these patients. The present study evaluates the oral fungal composition in AN and BN patients with the use of both culture-dependent and culture-independent methodologies.

MATERIALS AND METHODS

This study was approved by the local Ethics Committee (protocol PH/CEP 061/2006). The study was undertaken with the informed written consent of each subject.

Fifty-six female and 3 male patients, 19-58 years old (median 26 years), with eating disorders were included in the study. Samples were collected from 32 AN patients and 27 BN patients who were diagnosed in accordance with DSM-IV[3] and were under initial treatment at the Institute of Psychiatry, University of São Paulo. The AN patients were divided into 2 subtypes, purging (21 patients) and restrictive (11 patients).

The control group contained 59 individuals, matched to the ED group by age (±2 years; 18-57 years, median 26 years), sex, and oral conditions (use of dentures or orthodontic devices, smoking, and Decayed, Missing, and Filled Teeth index). These healthy volunteers were selected from patients treated at São José dos Campos Dental School. Patients with diabetes mellitus or other systemic diseases, pregnant women, total denture users, and individuals prescribed with antibiotics, antifungals, or oral rinses in the preceding 45 days were excluded.

Patient data were collected from the medical records. General oral health status examinations and anamnesis were performed under dental office conditions by the same researcher. The research of oral conditions commonly described among ED patients included the clinical diagnosis of perimolysis (classic dental erosion especially on the palatal face of the anterior and posterior teeth), oral ulcerations (that can be caused by the rapid ingestion of food or by the force of regurgitation in these patients), caries (clinically visible decayed teeth), gingivitis,[12] gingival recession (exposure in the dental roots caused by a loss of gum tissue and/or retraction of the gingival margin from the crown of the teeth), sialoadenosis (a noninflammatory enlargement of the salivary glands caused by a peripheral autonomic neuropathy, which is responsible for disordered metabolism and secretion, resulting in acinar enlargement and functional impairment), erosion (irreversible loss of enamel and dentine from the tooth due to chemical dissolution by acids not of bacterial origin[1,9]), and candidiasis.[1,9]

Oral rinses with sterilized phosphate-buffered saline solution (PBS, 0.1 mol/L, pH 7.2)[13] and supragingival biofilm samples[14] were collected. Marginal supragingival plaque was collected with sterile dental explorer from both the buccal surface of teeth 11, 31, 16, and 26 and the lingual surface of teeth 36 and 46, based on the Simplified Oral Hygiene Index.[15] The biofilm was transfered to a plastic tube containing 2 mL PBS.

Oral rinse samples (100 μL) were plated on Sabouraud dextrose agar (Himedia, India) supplemented with chloramphenicol (Inlab, Brazil; 0.1 mg/mL). Plates were incubated at 37°C for 48 hours, after which the number of colony-forming units per milliliter (cfu/mL) was calculated. Species identification was performed with the API 20 C Aux (Biomérieux, France), a standardized and miniaturized commercial system for identification of yeasts. Identification of *Candida dubliniensis* was confirmed by a multiplex polymerase chain reaction (PCR).[16,17]

Difference in the prevalence of yeasts in the oral cavity was analyzed using *Z* test. Results were expressed in cfu/mL and were compared with the use of analysis of variance and Mann-Whitney test ($\alpha = 5\%$). Statistical tests were used to compare the ED and control groups, the AN and BN groups, and the purging and restrictive subtypes of AN.

For molecular analysis, 100 μL of each oral rinse was transfered to a plastic tube containing 100 μL buffer solution (0.3 mol/L sucrose, 50 mmol/L Tris-HCl (pH 8.5), 1 mmol/L EDTA) and was stored at −80°C.

Five patients from each group were selected for evaluation by a culture-independent method. Oral rinses and supragingival biofilm samples were centrifuged at 7,500*g* for 15 minutes, and the supernatant was discarded. The pellets were suspended in 100 μL of a lysis solution (0.9 mol/L sorbitol, 0.1 mol/L EDTA, 50 mmol/L dithiothreitol (Fermentas, Foster City, CA); zymolyase 165 μg/mL (ICN Biochemicals, Irvine, CA). After incubation at 37°C for 30 minutes, 10 μL 10% sodium dodecyl sulfate was added, and the tubes were incubated at 65°C for 30 minutes. Precipitation was performed as described by Crouse and Amorese (1997).[18]

The D1/D2 region of the 28S rRNA gene was amplified under typical conditions with the use of a set of generic primers, as previously described by Donnelly et al. (1999).[16] Cloning of PCR-amplified DNA was performed with the TOPO TA cloning kit and *Escherichia coli* TOP 10 cells (Invitrogen) according to the manufacturer's instructions. Preparations of plasmid DNA (pDNA) for sequencing were obtained with the use of boiling procedures as well as an adaptation of the alkaline lysis procedure.[19,20]

Sequencing reactions followed the protocol provided by the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 (Applied Biosystems, Foster City, California). The reactions were analyzed with the use of the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Table I. *Candida* species observed in oral rinse samples from eating disorders (ED; n = 59) and control (n = 59) groups

	ED		Control		Total	
Species	*n**	*%*	*n**	*%*	*n**	*%*
Candida albicans	*120*	81.63	*51*	80.9	*171*	81.42
Candida dubliniensis	*8*	5.44	*3*	4.76	*11*	*5.23*
Candida parapsilosis	*6*	4.08	*0*	0	*6*	*2.85*
Candida glabrata	*4*	2.72	*3*	4.76	*7*	*3.33*
Candida zeylanoides	3	2.04	0	0	3	1.42
Candida tropicalis	4	2.72	3	4.76	5	2.38
Candida krusei	2	1.36	2	3.17	4	1.90
Candida guilliermondii	0	0	1	1.58	1	0.47
Total	147	100	63	100	210	100

*Number of isolates.

A total of 100 clones per subject were sequenced. Quality reads of at least 400 bp were used to determine the identity or approximate phylogenetic position. Full sequences were obtained for clones that were <98% similar to the closest known fungal sequences. The sequences were compared to those in the National Center for Biotechnology Information GenBank database using the BLASTN algorithm and were aligned using Clustal W.[21] Phylogenetic trees were constructed with the use of Mega 4 software.[22]

RESULTS

Oral examinations showed that 6.8% of the ED patients had perimolysis and that 1.7% had ulcerations in soft tissue. The most common observations were caries (50.8%), gingivitis (39%), gingival recession (25.4%), and abrasion (11.8%). Sialadenosis and erosion in the posterior teeth were not observed among these patients. One patient reported a history of oral candidiasis, and 27.1% reported burning mouth sensations. No patient presented with candidiasis lesions.

Self-induced vomiting (69.5%) was the most frequently reported compensative practice to prevent weight gain, followed by the use of laxatives (50.8%), diuretic drugs (32.2%), appetite suppressants (amphetamines or medicines for weight loss; 18.6%), and excessive physical exercising (6.8%).

Pharmacologic therapy for eating disorders varied among patients. The pharmaceutical regimen was established according to the clinical history and modified according to disease progression. Among the comorbidities, depression was the most frequently observed among patients (52.5%).

A greater percentage of individuals were positive for *Candida* species (74.6%) in the ED group compared with the control group (50.8%; $P = .006$). A total of 210 yeast isolates were obtained (147 from the ED group and 63 from the control group). Table I shows the species identified in each group.

Median values of cfu/mL of yeasts were higher in the ED group (median 987.5 cfu/mL, interquartile range [IQR] 3,513 cfu/mL) compared with the control group (median 12.5 cfu/mL, IQR 350 cfu/mL; $P = .000$). Comparison between the yeast counts among AN patients (n = 32) and BN patients (n = 27) was also performed. The distribution of the AN group (median 1,106.3 cfu/mL, IQR 3,444 cfu/mL) did not differ from the distribution of the BN group (median 575 cfu/mL, IQR 3,938 cfu/mL; $P = .9389$). In the AN group, statistical comparison was performed on the purging (n = 21) and restrictive (n = 11) subtypes, and the distribution of the purging group (median 1,225 cfu/mL, IQR 3,357 cfu/mL) did not differ from the restrictive group (median 1,275 cfu/mL, IQR 6,138 cfu/mL; $P = .9522$).

For the molecular analysis, 70 clones from the oral cavity and 30 from supragingival biofilms were generated for each patient. A total of 15 different species of fungi were identified. Five species were detected only in the ED group, 1 only in the control group, and 9 in both groups. Two uncultured species were detected in the ED group. Figure 1 shows the species found in each group by sampling site.

DISCUSSION

Nutritional deficiency and induced vomiting observed among ED patients can cause both systemic changes and alterations of the commensal oral microflora. The oral clinical alterations have been described in the literature, but little is known about the oral microbiota. Identification of microflora diversity is only the first step in understanding the ecologic functions of the fungi in the oral cavity.[23]

Oral infections by *Candida albicans* are common in people with impaired immune systems, and cases of severe immunosuppression may be related to systemic infection.[24,25] For the present study, an oral rinse method was chosen for sampling the microbiota as a whole, as described by Samaranayake et al. (1986).[13] A

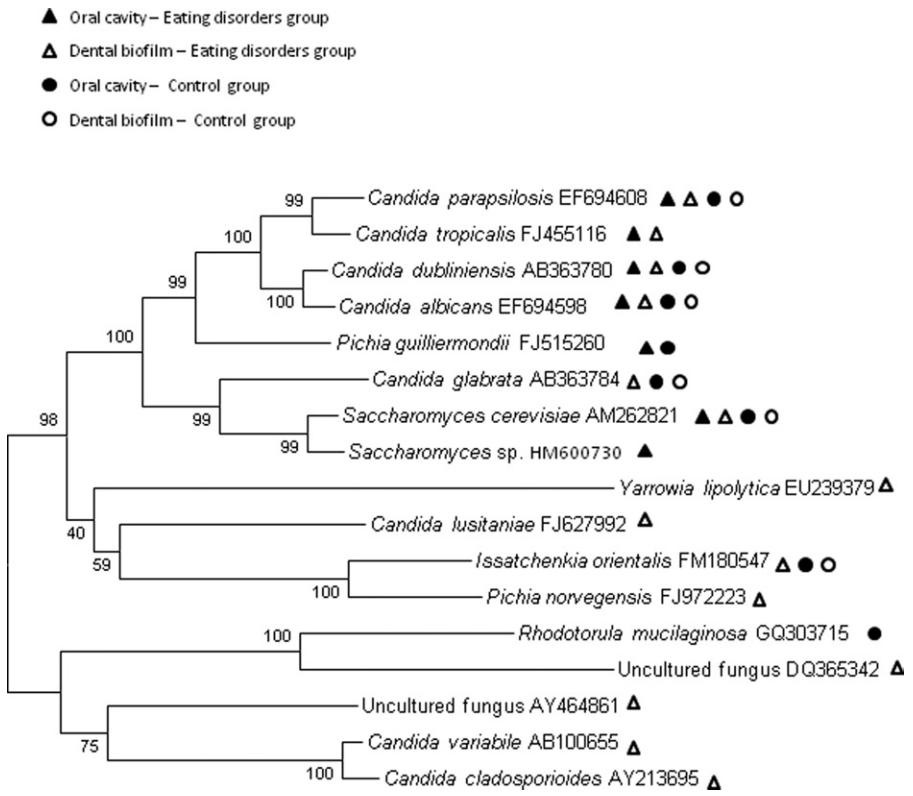

Fig. 1. Neighbor-joining tree based on the D1/D2 region of 28S rRNA showing the fungal species identified in each group (eating disorders group: n = 10; control group: n = 5) according to the sampling sites. Codes correspond to GenBank accession number, and the bootstrap values at each branching are based on 1,000 replicates.

determining factor for choosing this method was the difficulty in using other sampling methods. ED patients showed an increased regurgitation reflex, which was possibly due to the high frequency of self-induced vomiting to prevent weight gain. In addition, supragingival biofilm was collected for molecular analysis to augment the oral microbial evaluation.

In the present study, 74.6% of patients in the ED group were positive for *Candida* species. A lower frequency (50.8%) was observed among the control group. These results are similar to earlier studies, which reported that patients with certain predisposing factors showed a higher frequency of isolates compared with control groups. The most frequent systemic factors cited in the literature are the use of broad-spectrum antibiotics or steroids,[26,27] malnutrition,[28] diabetes mellitus,[29] and immunosuppression.[30,31] Poor oral hygiene, presence of dentures,[32] orthodontic appliances,[33] diet,[34] smoking,[35] and xerostomia[36] are considered to be important site-specific predisposing factors.[37]

Higher prevalence of isolates in the ED group may be caused by several factors. Vomiting decreases oral pH and some studies suggest that a more acidic environment favors colonization by *Candida* species.[30] Another factor, related to the bulimic diet during a compulsive crisis, is the high consumption of carbohydrates and sucrose.[1] Also, oral candidiasis has been associated with nutritional deficiency, including low levels of iron, zinc, vitamin K, and other water-soluble vitamins.[2] A statistically significant difference was observed between the ED and control groups, but not between AN and BN patients or between the purging and restrictive subtypes. We could not establish a direct relationship between any factor (systemic or oral) and the increased prevalence of fungi in oral cavity of these patients.

In studies of oral isolates, *C. albicans* is frequently reported as the most commonly isolate both in healthy subjects and in cases of candidiasis.[26] In this study, the ED group showed greater species diversity compared with the control group. Similar results were previously observed in patients with other predisposing factors. Belazi et al. (2005)[29] observed that the oral carriage of yeast was significantly higher and had greater species diversity in diabetic patients compared with healthy subjects. The same was observed by Back-Brito et al. (2009)[31] among HIV-positive patients. Also, heart transplant patients had significantly higher oral *Candida* titers than a control group.[38]

Although the prevalence of non-*albicans* species represented only 18.4% of the isolates in the ED group,

this result is relevant, because reports of infections caused by non-*albicans* species have increased significantly in recent years. Moreover, candidemia caused by *C. albicans* generally has a better prognosis compared with non-*albicans* species.[39,40]

Interestingly, *C. zeylanoides* was identified only in ED patients. This species is frequently associated with onychomycosis.[41] An earlier study reported that the presence of onychophagy is related to anxiety or self-aggression,[9] which could explain the presence of this species in patient oral samples. *C. zeylanoides* has been previously associated with candidemia and arthritis.[39]

Culture techniques are used to correlate microorganisms and diseases. These techniques demonstrate the presence of viable microorganisms. Also, virulence studies and susceptibility profiles to different antimicrobial agents are supported with this approach. However, there are some limitations in using these techniques alone. Fastidious microorganisms are difficult to culture by traditional microbiologic methods. These organisms can be identified with the use of molecular techniques. Therefore, the combination of phenotypic and molecular methods is the ideal approach to study microbial diversity, combining comprehensive and complementary information.

For molecular procedures, 5 patients from each group (anorexic, bulimic, and healthy subjects) were selected. Analysis was performed individually, without pooling the samples, to allow correlation of the results with dental and medical histories of each patient. The libraries were generated separately for each sample collected (oral rinses and dental biofilms). A total of 1,500 clones were analyzed.

The results obtained by molecular methods revealed the existence of fungal genera other than *Candida*. Part of this discrepancy is related to the fungi taxonomy and changes to it in recent decades, especially with the advent of molecular methodologies. Fungi may present in 2 distinct reproductive forms, sexual and asexual, which in the past resulted in a system of dual nomenclature for the 2 forms of the same species because one had been identified without recognition of the other reproductive form. The application of molecular techniques in fungal taxonomy has resulted in a tendency to unify the system of nomenclature based on rDNA sequence. This unified classification is gradually being implemented as species classified previously by classic microbiology in the genus *Candida* are now being reclassified, taking into account the sexual reproductive stage, such as *Clavispora lusitaniae*, *Issatchenkia orientalis*, *Pichia guilliermondii*, *Pichia norvegensis*, and *Yarrowia lipolytica*.[42]

Other genera were molecularly identified among the patients. *Cladosporium* is related to skin infections after traumatic and penetrating injuries. *Saccharomyces cerevisiae* and *Rhodotorula* are isolated from human skin and mucosa. Isolation of these species from the oral cavity is also reported in other studies using phenotypic identification in patients with different systemic conditions.[27,28,30] Aas et al. (2007),[43] using the technique for amplification of 18S rRNA gene, reported that, surprisingly, *S. cerevisiae* was the only fungal species found in an HIV-positive patient with linear gingival erythema and in 2 HIV-positive patients with periodontitis, and cited that this species has been associated with opportunistic infections in patients with severe immunosuppression. *C. albicans* was predominant in 2 periodontitis HIV-positive patients with low viral loads and high CD4 levels.

Recently, Ghannoum et al. (2010)[23] used pyrosequencing to obtain a more comprehensive profile of fungal flora. Sample collection was performed by oral rinses. A great variety of fungal microbiota were characterized in 74 genera of cultivable fungi and 11 non-cultivable fungi, which is a larger degree of diversity than we observed.

The identification of uncultured species first reported in the present study was also observed in dental biofilm from the ED group. The clinical relevance of these fungal species and their presence in the oral cavity is still unknown. Considering that oral *Candida* colonization is recognized to be a risk factor for infections in immunocompromised patients, these fungi may also be important as potential opportunistic pathogens. Within the limits of this study, it could be concluded that the ED group showed a higher prevalence of yeast and a greater variability of *Candida* species than the control group. Investigation using molecular methods revealed greater fungal diversity, besides *Candida* species in the groups studied. The isolation of unusual fungal species among these patients is clinically relevant because reports of infections caused by rare microorganisms have been on the rise among immunocompromised patients.

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